SeraMir™ Exosome RNA Amplification Kit

Cat. # RA800A/TC-1
Cat. # RA805A-1, RA808A-1
Cat. # RA806A/TC-1
Cat. # RA810A/TC-1
Cat. # RA820A/TC-1
Cat. # RA821A/TC-1

User Manual

See boxes for proper storage of the kit components upon receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 6)

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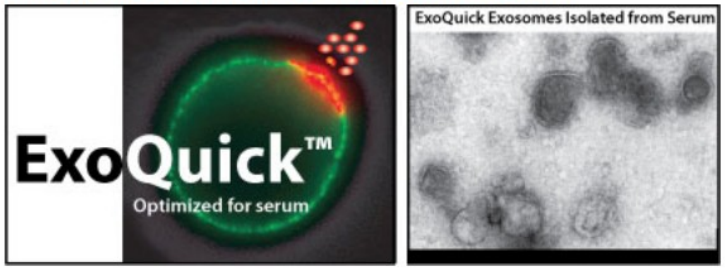
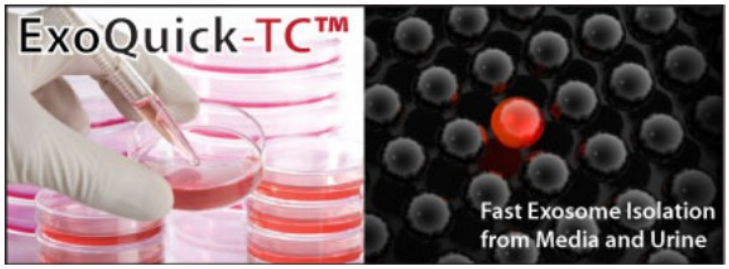
Precipitate Exosomes from Serum with ExoQuick



Isolate Exosomes from Culture Media and Urine with ExoQuick-TC



List of Components

RA800A/TC-1 Components	Amount
* ExoQuick (for 800A-1)	5 ml
* ExoQuick-TC (for 800TC-1)	10 ml
Lysis Buffer	8 ml
Wash Buffer	20 ml
Elution buffer	750 ul
SeraMir RNA Columns (RA800A-1)	20 columns
SeraMir RNA Columns (RA800TC-1)	10 columns
5x polyA Buffer	40 ul
$MnCl_2$ (25mM) solution	20 ul
ATP solution (5mM)	30 ul
PolyA polymerase	10 ul
SeraMir 3' Adaptor oligo	10 ul
5' Switch oligo	20 ul
SeraMir 5X RT Mix	80 ul
SeraMir Reverse Transcriptase	20 ul
SeraMir 2X Taq PCR Mix	250 ul
SeraMir PCR amplification primer mix	20 ul
3' SeraMir Reverse qPCR primer	600 ul

RA805A-1 Components	Amount
Control spike-in small RNA	100 ul
Control spike-in small RNA qPCR assay	100 assays
3' SeraMir Reverse qPCR primer	600 ul

RA806A/TC-1 Components	Amount
* ExoQuick (for RA806A-1)	5 ml
* ExoQuick-TC (for RA806TC-1)	10 ml
Lysis Buffer	8 ml
Wash Buffer	20 ml
Elution buffer	750 ul
SeraMir RNA Columns (RA806A-1)	20 columns
SeraMir RNA Columns (RA806TC-1)	10 columns

RA808A-1 Components	Amount
Lysis Buffer	8 ml
Wash Buffer	20 ml
Elution buffer	750 ul
SeraMir RNA Columns (RA806A-1)	20 columns

RA810A-1 Components	Amount
384 well SeraMir Profiler	20 profiles
3' SeraMir Reverse qPCR primer	600 ul

RA820A-1 Components	Amount
All of RA800A-1 or 800TC-1	20 reactions
All of RA805A-1	100 assays
All of RA810A-1	20 profiles

RA820TC-1 Components	Amount
All of 800TC and all of 806TC-1 but with 50 ml ExoQuick-TC	20 reactions
All of RA805A-1	100 assays
All of RA810A-1	20 profiles

The SeraMir™ kits are shipped on blue ice (-20ºC) especially for the cDNA synthesis reagents. The RNA columns and buffers can be stored at room temperature and the ExoQuick or ExoQuick-TC is stored at +4ºC. Please check each box for proper storage conditions upon receipt. Properly stored kits are stable for 1 year from the date received. The reaction size is based on using 500 µl serum with ExoQuick or for 5 ml media or urine using ExoQuick-TC for exosome isolation and exoRNAs amplification.

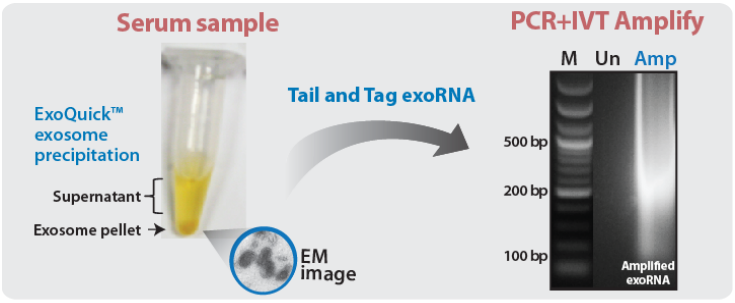
You will need:
100% Ethanol (molecular grade)

SeraMir™ Exosome RNA Amplification

A. Overview

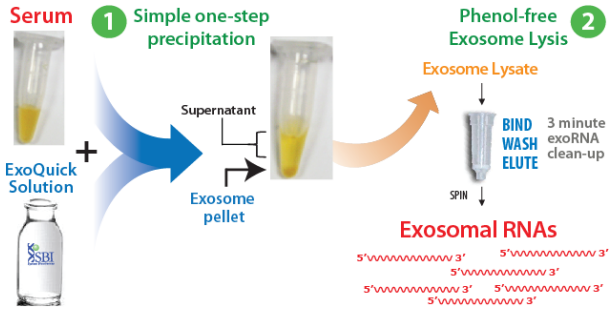
RNAs present in patient body fluids and cell culture media are a rich and untapped source of disease-related biomarkers. The RNAs are stable in serum because they are encapsulated in circulating exosomes. Exosomes are 40–100 nm membrane vesicles secreted by most cell types in vivo and in vitro. Exosomes are found in blood, urine, amniotic fluid, malignant ascite fluids, cell media and contain distinct subsets of microRNAs depending upon the tumor or tissue from which they are secreted. The SeraMir kit includes everything needed to accurately and sensitively measure RNAs from serum samples. Exosomes are efficiently isolated using SBI's ExoQuick solution, and the exoRNAs are purified using a phenol-free lysis buffer and rapid spin columns. The SeraMir kit enables the 3' tailing and simultaneous tagging of both 5' and 3' ends during cDNA synthesis - ready for qPCR. Primers for PCR amplification are included for highly sensitive applications.

- **No time-consuming ultracentrifugation to isolate exosomes**
- **Reduce variability - isolate exosomes first with ExoQuick (serum) or ExoQuick-TC (media)**
- **Increase sensitivity - amplify exoRNAs for qPCR**
- **Gain more data - use T7 IVT amplified sense exoRNAs for microarrays and NextGen sequencing**

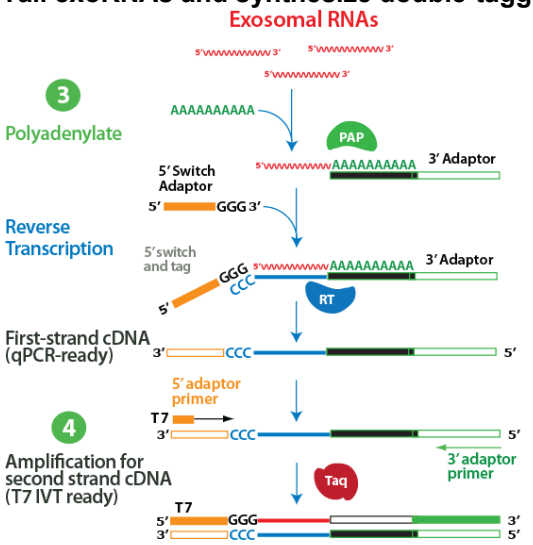


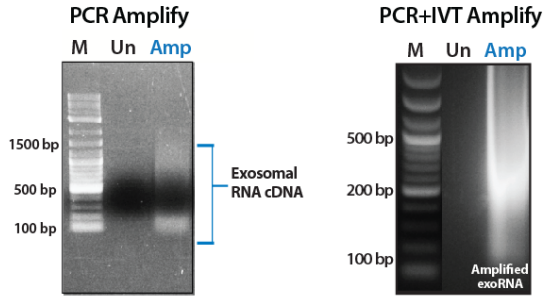
PROTOCOL AT A GLIMPSE

Precipitate serum exosomes and purify exoRNAs



Tail exoRNAs and synthesize double-tagged cDNA





B. Protocol

I. EXOSOME RNA ISOLATION PROTOCOL FROM 500µl SERUM or 5ml Media

* Collect biofluid and centrifuge at 3000 × g for 15 minutes to remove cells and cell debris.

Exosome Isolation and Lysis

1. Thaw serum sample on ice
2. Combine 500µl serum + 120 µl **ExoQuick**
   Or: 1 ml **ExoQuick-TC** with 5 ml Media
3. Mix well by inversion three times
4. Place at 4ºC for 30 minutes (serum) or for 6 hours to overnight (urine/media)
5. Centrifuge at 13,000 rpm for 2 minutes
6. Remove supernatant, keep exosome pellet
7. Add 350 µl **LYSIS Buffer** to exosome pellet and vortex 15 seconds
8. Place at room temperature for 5 minutes (to allow complete lysis)
   *--- optional--- add 5*µl *of SeraMir control RNA spike-in (cat#RA805A-1)*

exoRNA Purification

9. Add 200µl of 100% **Ethanol**, vortex 10 seconds
10. Assemble spin column and collection tube
11. Transfer all (600µl) to spin column
12. Centrifuge at 13,000 rpm for 1 minute
    (check to see that all flowed through,
    otherwise spin longer)
13. Discard flow-through and place spin column back into collection tube
14. Add 400µl **WASH Buffer**
15. Centrifuge at 13,000 rpm for 1 minute
16. Repeat steps 13 to 15 once again (total of 2 Washes)
17. Discard flow-through and centrifuge at 13,000 rpm for 2 minutes to dry (IMPORTANT !)

exoRNA Elution

18. Discard collection tube and assemble
    spin column with a fresh,
    RNase-free 1.5ml elution tube (not provided)
19. Add 30µl **ELUTION Buffer** directly to membrane in spin column
20. Centrifuge at 2,000 rpm for 2 minutes (loads buffer in membrane)
21. Increase speed to 13,000 rpm and centrifuge for 1 minute (elutes exoRNAs)
22. You should have recovered 30-40µl exosome RNA

The yield of RNA from isolated exosomes is different depending on the starting biofluid or the type of cells that were grown in culture. Different cell types secrete varying levels of exosomes. For serum, the level of RNA isolated from 500 µl is usually in the 500ng range and can be measured using an Agilent Bioanalyzer or a NanoDrop Spectrophotometer. The recovery from cell media varies depending the cell type and growth confluency.

II. EXOSOME RNA cDNA SYNTHESIS

1 Poly A reaction

Add:	Per reaction
exoRNA	5 µl (eluted from spin column)
5X polyA Buffer	2 µl
$MnCl_2$ (25 mM)	1 µl
ATP (5 mM)	1.5 µl
polyA polymerase	0.5 µl

Incubate at 37ºC for 30 minutes

2 Adaptor Anneal Reaction

Add 0.5 µl SeraMir 3' Adaptor Oligo
Incubate at 60ºC for 5 minutes
Incubate at Room temperature 2 minutes
Place on ICE

3 RT Reaction

Add:	Per reaction
polyA exoRNA	(10 µl from above)
5X RT Master Mix	4 µl
5' SeraMir Switch Oligo	1 µl
Reverse Transcriptase	1 µl
Water	4 µl
	20 µl TOTAL

Incubate at 42ºC for 30 minutes
Incubate at 95ºC for 10 minutes
HOLD at 15ºC

III. qPCR PROFILING OF exo-cDNA

(cat# RA805A-1 SeraMir Spike-in RNA qPCR assay and #RA810A-1 SeraMir Exosome RNA 384 microRNA qPCR Profiler)

To test your exo-cDNA, we recommend performing a qPCR assay for the RA805A-1 Spike-in RNA control or proceed to the 384 well SeraMir Profiler setup (qPCR array contains the Spike-in qPCR assay).

For 96-well plates:

Add:	Per well
exo-cDNA	0.5 µl from above
2X SYBR Master Mix *	15 µl
5' SeraMir Spike-in assay primer	1 µl
SeraMir 3' Reverse qPCR primer	0.5 µl
Water	13 µl
	30 µl TOTAL

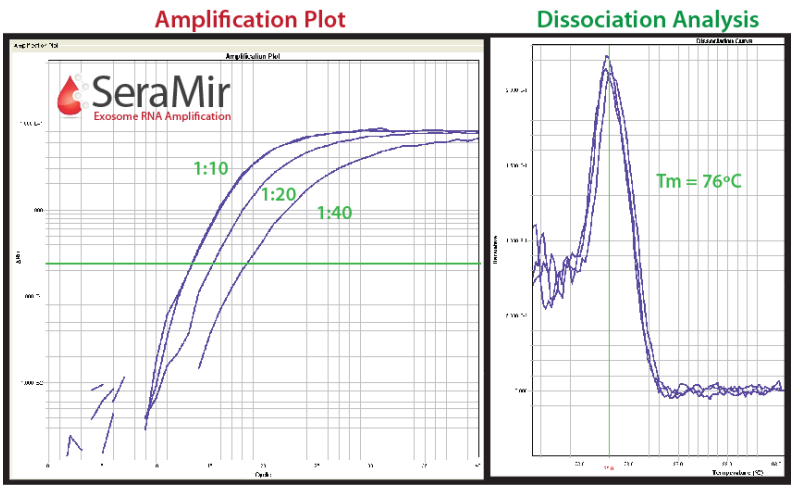
* SBI recommends Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221.

For 384-well plates:

Add:	Per well
exo-cDNA	1 µl (of 1:50 dilution)
2X SYBR Master Mix *	3 µl
5' SeraMir Spike-in assay primer	0.2 µl
SeraMir 3' Reverse qPCR primer	0.1 µl
Water	1.7 µl
	6 µl TOTAL

* SBI recommends Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221

Sample qPCR data for the SeraMir Spike-in RNA



If 5 µl of the SeraMir Spike-in RNA was used during the exoRNAs isolation, then you should expect to observe a Ct of about 15 to 20.

Setup of the 384 well SeraMir Profiler (cat# RA810A-1)

Mastermix qPCR Reaction Set up for ONE entire 384-well qPCR plate

To determine the expression profile for your miRNAs under study, mix the following for 1 entire 384 qPCR plate:

For 1 entire plate :

+	1150 µl	**2X SYBR Green*** qPCR Mastermix buffer
	39 µl	**3' SeraMir Reverse Primer (10µM)**
	5 µl	**User-synthesized SeraMir cDNA**
	1090 µl	**Nuclease-free water**
	2284 µl	Total

Aliquot 5µl of Mastermix *into every well* in your 384-well qPCR Plate

*** SBI has tested and recommends SYBR Green Master mix from three vendors:**
1. Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221
2. Power SYBR Master Mix® (Cat numbers 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems
3. SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat numbers 11760-100, 11760-500, and 11760-02K)

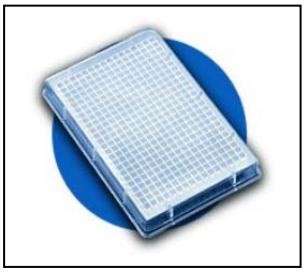
Resuspend the MicroRNA assay Primers with 22µl water in each well before use. Spin briefly to collect contents at bottom of wells.

Then :

Load 1µl per well of each of the Primers from the Primer Stock plate into your qPCR plate (well A1 into qPCR plate A1, etc.)

Once reagents are loaded into the wells, cover the plate with an optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

*** Use a Multichannel pipette to load the qPCR plate with MasterMix and Primers:** Pour the Mastermix into a reservoir trough and use a 8 or 12 channel pipette to load the entire 384-well qPCR plate with the Mastermix. Then load the primers from the primer plate to the qPCR plate using a separate multichannel pipette.



2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7900HT Real-time PCR System but can also apply to any other 384-well system. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from SBI's 7900HT Real-time instrument setup is shown below also. Default conditions are used throughout.

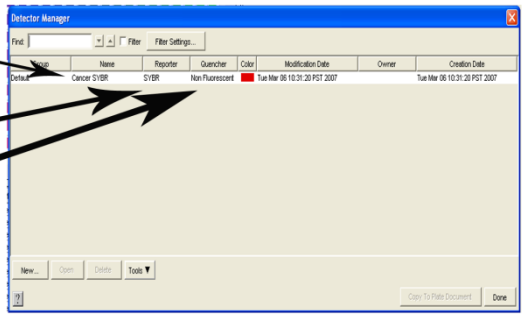
Create a detector:

1. Create a new Detector

2. Name the Detector (any name will do)

3. Select Reporter Dye as "SYBR Green"

4. Select Quencher Dye as (none)



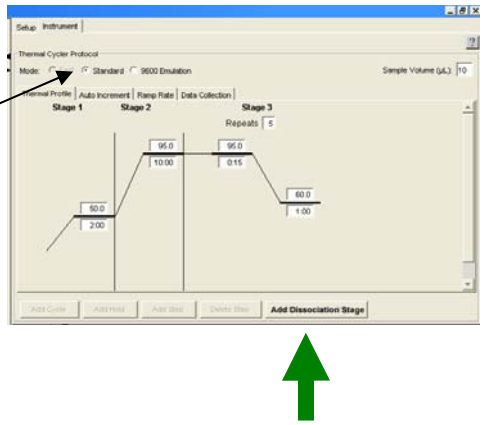
Instrument setup:

qPCR cycling and data accumulation conditions:

Standard Protocol

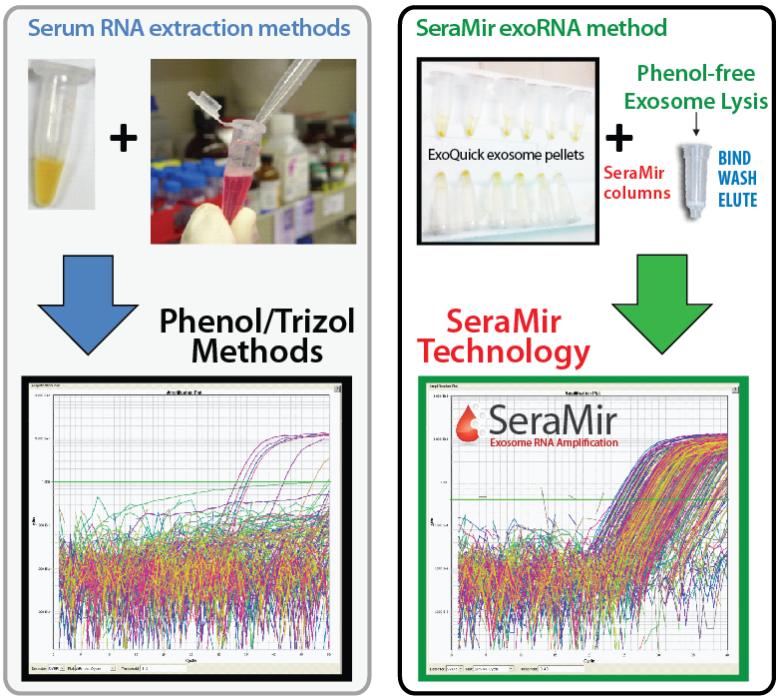
1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of Stage 3), data read at 60°C 1 min. Step.



An additional recommendation is to include a **Dissociation Stage** after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

Sample 384 well SeraMir Profiler Data



The results are clear – obtain more data with SeraMir.

Serum RNA prepared by conventional Trizol versus the SeraMir kit. Profiling of 380 Human microRNAs across the SeraMir 384 Profiler (cat# RA810A-1). The phenol-free exosome lysis step coupled to the small RNA binding columns isolates exoRNAs with much higher purity than Trizol/Phenol based methods. The SeraMir exoRNAs are compatible with downstream polyadenylation and reverse trancription reactions for amplification and accurate qPCR profiling.

IV. EXOSOME cDNA AMPLIFICATION

The first-strand exoRNAs cDNA made in STEP II. Can be amplified to make double-stranded exo-cDNA compatible with T7 in vitro transcription reactions to make amplified "sense" RNAs that work with most microRNA microarrays and can be adapted to use with NextGen sequencing preparation protocols.

Add:	Per reaction
exoRNA amplified cDNA	(2 µl from above)
2X PCR Master Mix	12.5 µl
5' SeraMir PCR Primer Mix	1 µl
Water	9.5 µl
	25 µl TOTAL

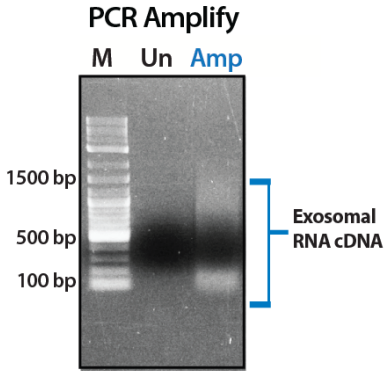
Place the reactions in a thermal cycler, and cycle using the following program:

- 95°C for 5 min
- 95°C for 25 sec
- 60°C for 20 sec
- 72°C for 30 sec

35 Cycles

- 72°C for 30 sec
- 15°C hold

Visualize the PCR products on a 1.5% agarose gel, load 5 µl per well.



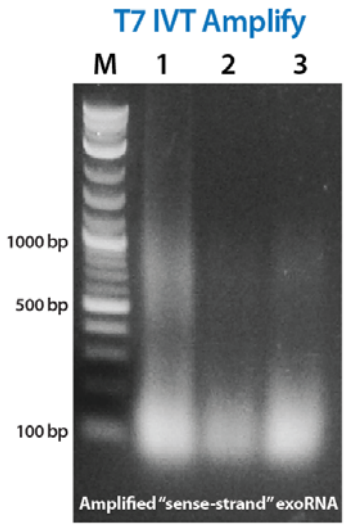
V. EXOSOME SENSE RNA AMPLIFICATION

SBI recommends using Epicentre's AmpliScribe™ T7-Flash™ Transcription Kit, catalog# ASF3257.

4.3 µl RNase-Free water
2 µl Amplified exo-cDNA (from STEP IV.)
2 µl AmpliScribe T7-Flash 10X Reaction Buffer
1.8 µl 100 mM ATP
\+ 1.8 µl 100 mM CTP
1.8 µl 100 mM GTP
1.8 µl 100 mM UTP
2 µl 100 mM DTT
0.5 µl RiboGuard RNase Inhibitor
2 µl AmpliScribe T7-Flash Enzyme Solution
20 µl Total reaction volume

Incubate at 45ºC for 45 minutes.

Visualize the PCR products on a 1.5% agarose gel, load 5 µl per well.



The RNA sizes will range from 80 bases to as long as 1kb. The SeraMir adaptors add 62 bases to the sizes of the exoRNAs, thus a base T7 IVT product corresponds to an exoRNAs insert sequence of about 20 bases.

C. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

II. Licensing and Warranty Statement

Limited Use License

Use of the SeraMir™ Exosome RNA Amplification Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.

This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein.

Limited Warranty

SBI warrants that the Product meets the specifications described in this manual. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.
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